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Spectrum of Mutations of the *AAAS* Gene in Allgrove Syndrome: Lack of Mutations in Six Kindreds with Isolated Resistance to Corticotropin

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Familial glucocorticoid deficiency due to corticotropin (ACTH) resistance consists of two distinct genetic syndromes that are both inherited as autosomal recessive traits: isolated ACTH resistance (iACTHR), which may be caused by inactivating mutations of the ACTH receptor (the MC2R gene) or mutations in an as yet unknown gene(s), and Allgrove syndrome (AS). The latter is also known as triple-A syndrome (MIM 231550). In three large cohorts of AS kindreds, the disease has been mapped to chromosome 12; most recently, mutations in the *AAAS* gene on 12q13 were found in these AS families. *AAAS* codes for the WD-repeat containing ALADIN (for alacrima-achalasia-adrenal insufficiency-neurologic disorder) protein. We investigated families with iACTHR (n = 4) and AS (n = 6) and a Bedouin family with ACTHR and a known defect of the TSH receptor. Four AS families were of mixed extraction from Puerto Rico (PR); most of the remaining six families were Caucasian families from North America (NA). Sequencing analysis found no *MC2R* genetic defects in any of the kindreds. No iACTHR kindreds, but all of AS families, had *AAAS* mutations. The previously reported IVS14+1G→A splice donor mutation was found in all PR families, apparently due to a founder effect; one NA kindred was heterozygous for this mutation. In the latter family, long-range PCR failed to identify a deletion or other rearrangements of the *AAAS* gene. No other heterozygote or transmitting parent had any phenotype that could be considered part of AS. The IVS14+1G→A mutation results in a premature termination of the predicted protein; although it was present in all PR families (in the homozygote state in three of them), there was substantial clinical variation between them. One PR family also carried a novel splice donor mutation of the *AAAS* gene in exon 11, IVS11+1G→A; the proband was a compound heterozygote. A novel point mutation, 43C→A(Gln^{15}Lys), in exon 1 of the *AAAS* gene was identified in the homozygote state in a Canadian AS kindred with a milder AS phenotype. The predicted amino acid substitution in this family is located in a sequence that may participate in the preservation of stability of ALADIN β-strands, whereas the splicing mutation in exon 11 may interfere with the formation of WD repeats in this molecule. We conclude that 1) *AAAS* does not appear to be frequently mutated in families with iACTHR; 2) *AAAS* is mutated in AS families from PR (that had previously been mapped to 12q13) and NA; and, 3) there is significant clinical variability between patients with the same *AAAS* defect. (*J Clin Endocrinol Metab* 86: 5433–5437, 2001)

ALLGROVE OR TRIPLE A syndrome (AS; MIM 231550) (1) is an autosomal recessive condition associated with adrenal insufficiency due to ACTH resistance, alacrima, and achalasia; it was first described in 1978 (2). Other features of AS include autonomic and/or peripheral neuropathy, hyperkeratosis and delayed wound healing, mental retardation and dementia, and, rarely, short stature (3–9). The complexity and variable presentation of the disorder led to a suggestion that the syndrome might be due to a contiguous gene deletion (3, 10). However, in several consanguineous families of European descent and in four Puerto Rican families with no known consanguinity, AS was mapped to 12q13 by linkage analysis (11, 12); genetic analysis in these kindreds did not suggest the presence of microdeletions.

In the search for the gene responsible for AS, a variety of genes were excluded in the 0.5-cM interval between polymorphic markers *D12S361* and *D12S368* (13, 14). The gene coding for the neuronal sodium channel *SCN8A* had previously been mapped to a clone containing *D12S368* (15), placing it at the AS locus. *SCN8A* is widely expressed in brain and spinal cord and in the peripheral nervous system (16), and three allelic mutations of *SCN8A* in the mouse cause a variety of neurologic abnormalities, including paresis, paralysis, ataxia, tremor, and dystonia (17). However, haplotype analysis placed the *SCN8A* gene telomeric to the AS candidate region (14).

Recently, the 12q13 region containing the AS gene was narrowed by homozygosity mapping in families of Middle

Abbreviations: ALADIN, Alacrima-achalasia-adrenal insufficiency-neurologic disorder; AS, Allgrove syndrome; B, Bedouin; iACTHR, isolated ACTH resistance; DHPLC, denaturing HPLC; NA, North American; PR, Puerto Rico; TSHR, TSH resistance.

Eastern and southern European extraction (18). The *AAAS* gene, which codes for the previously unknown protein ALADIN (alacrima-achalasia-adrenal insufficiency-neurological disorder), was mutated in the homozygote or compound heterozygote state in all kindreds with AS studied to date, including a large population collected worldwide (19, 20).

In the present study we investigated our patients with AS from North America (NA) and Puerto Rico (PR) for mutations of the novel gene. In addition, we examined the hypothesis that six of our kindreds with isolated corticotropin (ACTH) resistance (iACTHR) with no mutations of the *MC2R* gene and absence of other clinical stigmata of AS may also harbor mutations of the AAAS gene. Finally, one large inbred family from the Middle East with combined ACTHR and TSH resistance (21), was also screened for *AAAS* mutations.

Subjects and Methods

Patient population and DNA preparation

The review boards of participating institutions approved the contact of families with AS and the participation of patients and their relatives in the present study after informed consent was obtained. We studied families with iACTHR (n = 4), a highly inbred family with ACTHR and TSH resistance (ACTHR/TSHR) that has been described previously (21), and kindreds with AS (n = 6). Four of the AS families were of mixed Hispanic extraction from PR, and their clinical presentation has been described previously (12, 22); the remaining two families were Caucasian families from NA. Kindreds with iACTHR were from the United States (2), including one that we described previously, family HGD2 (22); one family was from Canada; and another was a Palestinian family from Israel. The inbred ACTHR/TSHR kindred was a Bedouin (B) family; in some of its members ACTHR and TSHR cosegregated; in others, iACTHR was present. Although the TSH receptor gene was found to be mutated (leading to truncation of the predicted peptide product), the MC2R locus was excluded by linkage analysis (21).

A clinical summary of all probands and their main manifestations other than ACTH resistance are presented in Table 1. A total of 34 subjects (including the probands, siblings, and parents when they were available; only the proband with ACTHR from the B family was included here) were studied. The diagnoses of achalasia and alacrima were made on the basis of clinical history, physical examination, and imaging studies. A 1-h ACTH (250 μg cortrosyn) stimulation test was performed in all subjects; the diagnosis of adrenal insufficiency was made by standard criteria.

DNA preparation and studies of the ACTHR (MC2R gene)

All patients gave informed consent to the participating institutions. DNA was prepared from collected blood samples as previously described (22, 23). A 1211-bp fragment of the ACTHR (the MC2R gene)-coding sequence (including flanking intronic sequence) was amplified with the 5′-ACTHR-1 (5′-GCAATAAACATACATGTGCAGGCT-3′) and the 3′-ACTHR-1 (5′-CTTGGCAACGTTATTCCCATGGA-3′) primer set as previously described (22). The 1211-bp PCR-amplified DNA fragment of the MC2R gene was then sequenced by asymmetric PCR as described previously (22).

Sequence analysis of the AAAS gene

The primers used for PCR amplification of the 16 coding exons of the *AAAS* gene and surrounding intronic sequences are available from the authors upon request; the intron-exon boundaries of the gene have been published previously (20). Denaturing HPLC (DHPLC) was performed using the HELIX system (Varian, Inc., Walnut Creek, CA) as previously described (23–25). Runs were performed at temperatures recommended by the DHPLC Melt program (25). Samples that produced aberrant DHPLC traces were selected for further study. After purification of the PCR products (GFX, AP Biotech, Piscataway, NJ), samples were subject to sequencing using the BigDye terminator kit (PE Biosystems, Foster City, CA) and were analyzed on an ABI 377 sequencer (PE Biosystems, Foster City, CA), as previously described (23).

Traces were analyzed with Sequencher 3.0 (Genecodes, Ann Arbor, MI; Fig. 1). All sequence alterations were confirmed by both forward and reverse strand sequencing (23) Restriction digestion was also used for confirmation whenever that was possible, as described previously (19).

Long-range PCR of the AAAS gene

For exclusion of deletions or other intragenic rearrangements long-range PCR of the AAAS gene was performed using primers 1F and 7R, 1F and 16R, and 9F and 16R (Fig. 2). The conditions of these reactions were identical to those used for the amplification of the individual exons, which have been described previously (19).

Results

Clinical analysis

All probands (n = 13) were affected with ACTH unresponsiveness (four, eight, and one from the iACTHR, AS, and ACTHR/TSHR kindreds, respectively). All of the AS patients had alacrima; six had achalasia. Neurological dysfunction (autonomic and sensory or motor nervous system abnormalities) was present in three AS patients; learning

TABLE 1. Patients affected by ACTHR and/or AS

Patient no./sex	Family	Country	Manifestations	Ref. no.	*MC2R* gene	*AAAS* gene
1/F	HGD1	CAN	Al, Ach, ACTHR, LD, ND	22	Normal	43C>A (Gln^{15}Lys) (Hom)[a]
2/F	AS1	PR	Al, Ach, ACTHR, Sz	12, 22	Normal	IVS14+1G>A (Hom)
3/F	AS1	PR	Ach, ACTHR	12, 22	Normal	IVS14+1G>A (Hom)
4/M	AS2	PR	Al, ACTHR, MI, Sz, Mr	12, 22	Normal	IVS14+1G>A (Het) IVS11+1G>A (Het)[a]
5/F	AS3	PR	Al, ACTHR	12, 22	Normal	IVS14+1G>A (Hom)
6/F	AS4	PR	Al, Ach, ACTHR, ND	12, 22	Normal	IVS14+1G>A (Hom)
7/F	AS4	PR	Ach, ACTHR	12, 22	Normal	IVS14+1G>A (Hom)
8/M	AL05	USA	Al, Ach, ACTHR, LD, ND	unpub	Normal	IVS14+1G>A (Het)
9/F	B	ISL	ACTHR	21	Normal	Normal
10/M	R01	USA	iACTHR	unpub	Normal	Normal
11/F	HGD2	AUS	iACTHR	22	Normal	Normal
12/M	R07	ISL	iACTHR	unpub	Normal	Normal
13/M	R09	USA	iACTHR	unpub	Normal	Normal

F, Female; M, male; Al, alacrima; Ach, achalasia; ACTHR, ACTH resistance; LD, learning disabilities; MI, mineralocorticoid deficiency; Mr, mental retardation; ND, neurologic deficiencies; Sz, seizures; unpub, unpublished; Hom, homozygote; Het, heterozygote. Countries: AUS, Australia; CAN, Canada; ISL, Israel; PR, Puerto Rico.

[a] New mutations of the *AAAS* gene are *underlined*.

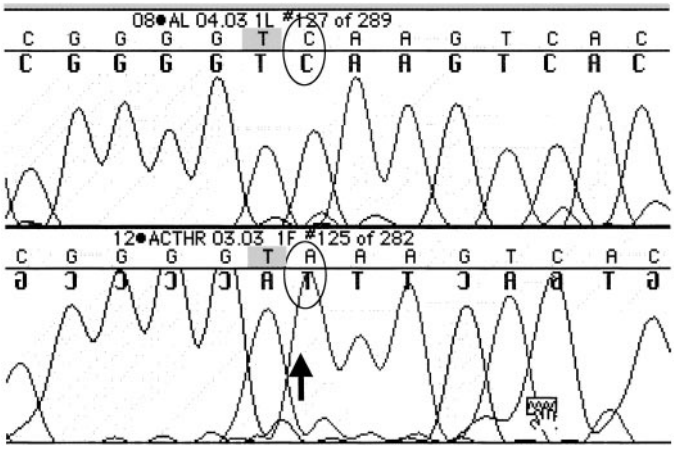

FIG. 1. A previously unreported homozygous missense mutation in kindred HGD1 (*lower panel*): 43 C→A changing Gln (CAA) to Lys (AAA) in codon 15 of the ALADIN protein. The normal sequence is shown in the *upper panel*.

disabilities or mental retardation were also present in three AS patients. Mineralocorticoid deficiency was present in two AS patients. None of the iACTHR patients had any other clinical conditions. The B family has been extensively described previously (21).

Genetic analysis

There were no mutations of the *MC2R* gene in any individuals. The entire coding region was analyzed, including the flanking intronic regions (from nucleotide −69 to 912 of the MC2R gene).

No iACTHR kindreds, but all AS families, had *AAAS* mutations. The previously reported IVS14+1G→A splice donor mutation (19) was found in all PR families (n = 4) in the homozygote (n = 3) or heterozygote state (n = 1); one additional kindred from NA was heterozygote for this mutation (Table 1, patient 8). In this kindred, long-range PCR failed to identify deletions or other rearrangements of the *AAAS* gene (Fig. 2). One PR family (Table 1, patient 4, family AS2) also carried a novel splice donor mutation of the *AAAS* gene in exon 11, IVS11+1G→A (data not shown); the proband was a compound heterozygote for this and the IVS14+1G→A mutation. A novel point mutation, 43C→A ($Gln^{15}Lys$), in exon 1 of the *AAAS* gene was identified in a Canadian family with AS (Table 1, patient 1, kindred HGD1; Fig. 1).

Genotype-phenotype analysis

With the exception of patient 8, all patients with a phenotype of AS had mutations of the *AAAS* gene in the homozygote or compound heterozygote state consistent with an autosomal recessive inheritance of the syndrome. None of the parents or heterozygote siblings (n = 34) tested by ACTH stimulation tests (n = 15) or other testing met any criteria for diagnosis of iACTHR or AS (data not shown).

The IVS14+1G→A mutation results in a premature termination of the predicted protein; although it was present in all PR families, there was substantial clinical variation between them. The proband of the AS2 family was a compound

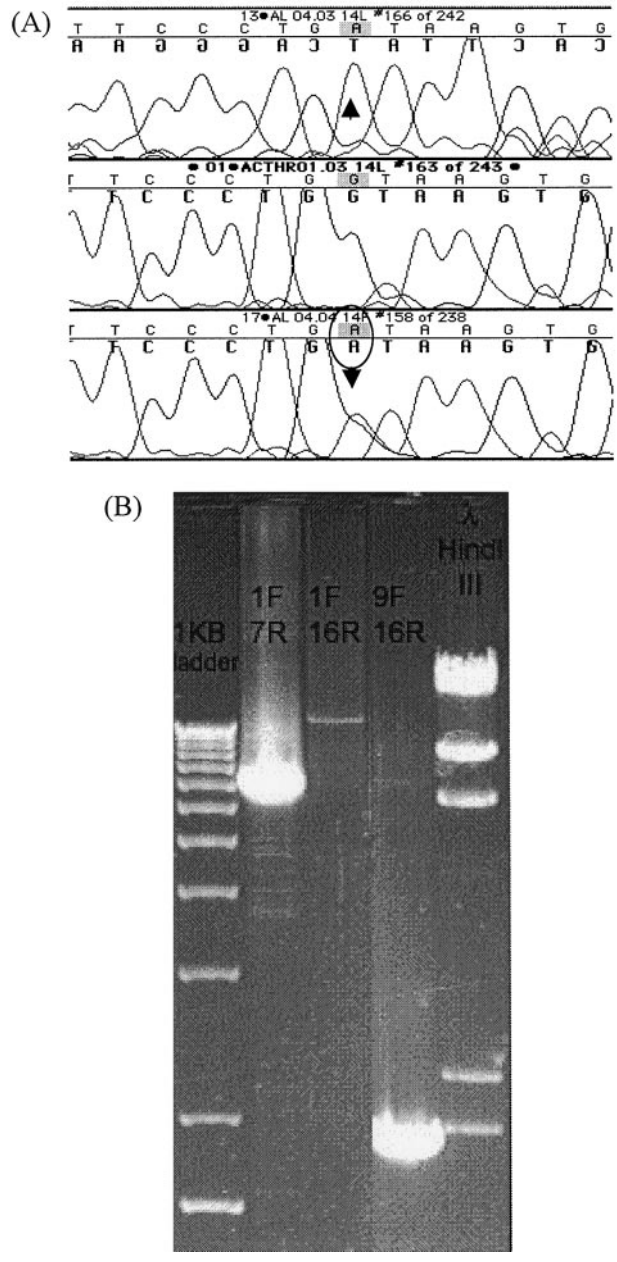

FIG. 2. A, Three sequence traces from the intron-exon 14 boundary of the *AAAS* gene. The *upper trace* shows the mutant sequence (IVS14+1G→A), the most common alteration of the *AAAS* gene described to date. The trace in the *middle* shows the normal sequence, and that in the *bottom* is from patient 8 (Table 1, kindred AL05), who was a heterozygote for that mutation. B, Long-range PCR of the *AAAS* gene in that individual failed to show any gross alterations of the *AAAS* gene, as demonstrated by the panel on the *left*. Three overlapping segments of the *AAAS* gene have the expected sizes (for the amplicons 1F and 7R, 1F and 16R, and 9F and 16R, they are 7,368, 14,210, and 2,006 bp, respectively).

heterozygote for that and another novel splicing mutation; his phenotype was more severe (Table 1). However, it is uncertain whether this is related to his genotype, as seizures and mental retardation may occur in patients with AS as a result of un- or undertreated adrenocortical insufficiency.

The novel point mutation, 43C→A($Gln^{15}Lys$), in exon 1 of

the *AAAS* gene was identified in a Canadian AS kindred with a slower onset of the individual manifestations of the AS; this patient was included in the paper by Wu *et al.* (22) as a kindred with iACTHR, because at the time she was not known to have the other components of the syndrome. The predicted amino acid substitution in this family is located in a sequence that may participate in the preservation of stability of ALADIN β-strands, whereas the splicing mutations in exons 11 and 14 may abolish the formation of WD repeats in this molecule.

Discussion

Cloning of the *AAAS* gene led to its screening in the available cohorts of patients with AS worldwide (19, 20). As in the families from PR in this study, the IVS14+1G→A mutation is the most common *AAAS* mutation. In our PR patients, as in the Middle Eastern and southern European population studied by Pelet-Tullio *et al.* (19), the frequent presence of this mutation is the result of a founder effect. Homozygosity was present in three of the four PR families (12), and the alleles of the chromosome bearing the IVS14+1G→A mutation in the fourth family were also identical (12, 14). New mutations have also been found, however, in ethnically diverse or mixed populations (this report and Ref. 20); the two new mutations presented in this paper in families HGD1 and AS3 (Table 1) appear to occur in chromosomes of a different lineage.

The present report adds to the list of mutations in the *AAAS* gene (19, 20), albeit with little, if any, genotype-phenotype correlation. More information on the effect of the individual genetic defects will have to wait until the function of the *AAAS* gene is clarified. Currently, ALADIN is postulated to be involved either in cytoplasmic trafficking (19), like other proteins with WD repeats, or in peroxisomal activities (20).

It is noteworthy that one patient was found to be a heterozygote only for the common *AAAS* mutation (IVS14+1G→A). Extensive sequencing and long-range PCR failed to identify other mutations or gene rearrangements. From the data in this paper and those in other reports (19, 20), there is little, if any, doubt that AS is an autosomal recessive disorder; none of the heterozygote siblings or the parents of our patients available for study were found to have any clinical phenotype of AS or even partial iACTHR. We have to assume that another mutation in patient 8 (kindred AL05) lies in the noncoding sequences of the gene and affects its expression and/or function.

It appears that families with iACTHR do not harbor *AAAS* mutations frequently. This fact and the absence of any symptoms in heterozygote carriers suggest that penetrance of the identified *AAAS* gene defects is close to 100% for AS (albeit with variable clinical expression). This study, however, does not rule out the possibility of functional defects of the *AAAS* gene in iACTHR; it is also possible that more subtle genetic changes in the *AAAS* gene may account for the clinical phenotype in a small number of iACTHR kindreds (26).

We conclude that AS, an autosomal recessive disorder, is caused by mutations of the *AAAS* gene in the third largest patient cohort reported to date; the present study also suggested that, in most cases, iACTHR without MC2R gene mutations is not a *forme fruste* of AS, although this finding needs to be confirmed in a larger study of patients with familial glucocorticoid deficiency.

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